Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity

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Abstract

***Background and aims*—The gastrointestinal microflora exerts a barrier effect against enteropathogens. The aim of this study was to examine if bifidobacteria, a major species of the human colonic microflora, participates in the barrier effect by developing antimicrobial activity against enterovirulent bacteria.**

***Methods*—Antibacterial activity was examined in vitro against a wide range of Gram negative and Gram positive pathogens. Inhibition of *Salmonella typhimurium* SL1334 cell association and cell invasion was investigated in vitro using Caco-2 cells. Colonisation of the gastrointestinal tract in vivo by bifidobacteria was examined in axenic C3/He/Oujco mice. Antimicrobial activity was examined in vivo in axenic C3/He/Oujco mice infected by the lethal *S typhimurium* C5 strain.**

***Results*—Fourteen human bifidobacterium strains isolated from infant stools were examined for antimicrobial activity. Two strains (CA1 and F9) expressed antagonistic activity against pathogens in vitro, inhibited cell entry, and killed intracellular *S typhimurium* SL1344 in Caco-2 cells. An antibacterial component(s) produced by CA1 and F9 was found to be a lipophilic molecule(s) with a molecular weight of less than 3500. In the axenic C3/He/Oujco mice, CA1 and F9 strains colonised the intestinal tract and protected mice against *S typhimurium* C5 lethal infection.**

***Conclusion*—Several bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity, suggesting that they could participate in the "barrier effect" produced by the indigenous microflora.**

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Keywords: bifidobacteria; infant microflora; gastrointestinal infection; antimicrobial; microbial infection; intestinal cells

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The normal flora of the human gastrointestinal tract contains many diverse populations of bacteria which play an essential role in the development and well being of the host.[1] In particular, the intestinal microflora exerts a protective role against pathogens.[2] Knowledge of the predominant genera and species, and their levels and biochemical activity are essential to understand the microbial ecology of the gastrointestinal tract. A long established but controversial concept is that of beneficial species.[3 4] Among the species present in the human intestinal microflora, several reports have emphasised the role of bifidobacteria. Bifidobacteria are anaerobic, rod shaped, Gram positive bacteria that are normal inhabitants of the human colon constituting a predominant part of the anaerobic flora. Indeed, bifidobacteria are the predominant intestinal organisms of breast fed infants.[5–8] Adults also carry bifidobacteria in their colonic flora.[9] The composition of the intestinal human gut microflora can be modulated by live microbial feed supplements.[10 11] Moreover, prebiotics—that is, non-digestible food ingredients—can also modify the intestinal microflora and in particular increase the level of bifidobacteria.[11 12] A role for bifidobacteria in host resistance to infection has been proposed.[13]In vitro laboratory and animal studies have shown that bifidobacteria exert antagonistic activity against pathogens.[14–20] Moreover, it is recognised that the antimicrobial properties of bifidobacteria could contribute to the protection that breast feeding provides against gut infection.[21 22] To gain further insight into the mechanism by which resident bifidobacteria of the human microflora could exert a protective role against pathogens, we examined the antibacterial activity of bifidobacterium strains isolated from infant stools.

Materials and methods

BACTERIA

Bifidobacterium strains were isolated from infant human stools. A nut sized piece of faeces was placed in a sterile tube. For optimal survival of the extremely sensitive anaerobic bacteria, the samples had to be treated within 30 minutes after emission. Otherwise, the samples were kept in an anaerobic jar until analysis (maximum of 10 hours). Isolation was conducted in an anaerobic Freter chamber. Firstly, a 10-fold dilution was performed in a pre-reduced Ringer solution with 10% glycerol. The sample was then aliquoted and a safety stock was prepared for freezing in liquid nitrogen. Serial dilutions were prepared and 100 µl of each dilution were plated on agar plates prepared with a medium selective for bifidobacteria.[23] Plates were incubated for two days under anaerobic conditions. Bifidobacterium colonies were round and white. However, other bacteria such as lactobacilli can grow on

Abbreviations used in this paper: SCS, spent culture supernatant; PBS, phosphate buffered saline; cfu, colony forming units; MRS broth, De Man, Rogosa, Sharpe broth; TSA, triptic soy agar.

this medium. To differentiate bifidobacteria from other colonies, it was necessary to examine each isolated colony by light microscopy. The selective medium used is particularly adapted to promote the typical Y shape of bifidobacteria. Likely colonies of bifidobacteria were isolated and grown again on the same medium for amplification, further identification, and conservation. Final identification was made using API tests (Api ID 32A. Bio Mérieux, Marcy l'Etoile, France). Colonies thus identified were extracted from the agar plates and homogenised in BHI medium (Oxoid) containing 40% glycerol (w/v). Aliquots were transferred in cryotubes. These tubes were frozen and kept in liquid nitrogen.

Before use, bifidobacteria were grown under anaerobic conditions (Gaspack H_2+CO_2) in De Man, Rogosa, Sharpe (MRS) broth (Biokar, Pantin France) 2×24 hours at 37°C. Spent culture supernatant (SCS) of bifidobacteria was obtained by centrifugation at 10 000 *g* for 30 minutes at 4°C. Centrifuged SCS was passed through a sterile 0.22 μm filter unit Millex GS (Millipore, Molsheim, France). Filtered SCS was verified for the absence of bifidobacteria by plating on tryptic soy agar. A pH ranging from 4 to 4.5 was observed for different bifidobacteria-SCS; consequently, the pH of bifidobacteria-SCS was adjusted to 4.5 with HCl for all experiments. Concentrated bifidobacteria-SCS was obtained by freeze drying (2.5-fold concentrate, pH 4.5).

Salmonella typhimurium SL 1344 was a gift from BAD Stocker (Stanford, California, USA),[24] *S typhimurium* C5 was provided by MY Popoff (Institut Pasteur, Paris, France),[25] *Listeria monocytogenes* EGD [HLY^+] was provided by J L Gaillard (Faculté Necker-Enfants Malades, Paris, France),[26] *Escherichia coli* C1845 was a gift from S Bilge (University of Washington, Seattle, USA),[27] *and Shigella flexneri* M90T was provided by P Sansonetti (Institut Pasteur, Paris).[28] *Clostridium difficile* Cd 79-685 was isolated from a stool sample of a patient with antibiotic associated pseudomembranous colitis (Institut de Bactériologie, Strasbourg, France).[29] *Staphylococcus aureus*, *Streptococcus D*, *Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* were stock clinical isolates from the microbiological laboratory of the Faculté de Pharmacie Paris XI, Châtenay-Malabry, France.

ANTIMICROBIAL TESTING

Antimicrobial activity of bifidobacteria was examined as previously described.[30–32] As indicator strain, *S typhimurium* SL1344 was grown overnight for 18 hours at 37°C in Luria broth. To obtain mid-logarithmic phase organisms, 10 ml of fresh trypticase soy broth were inoculated with 200 μl of cultured Luria broth and incubated for an additional three hours at 37°C. The bacteria were centrifuged at 5500 *g* for five minutes at 4°C, washed once with phosphate buffered saline (PBS), and resuspended in PBS. *S typhimurium* were counted and a volume containing 10^8 colony forming units (cfu)/ml was used to determine the activity of bifidobacteria-SCS. Colony count assays were performed by incubating 1 ml of PBS containing *S typhimurium* (10^8 cfu/ml) with 1 ml of bifidobacteria SCS at 37°C. At predetermined intervals, aliquots were removed, serially diluted, and plated on trypticase soy agar (TSA) to determine bacterial colony counts.

CHARACTERISTICS OF BIFIDOBACTERIA-SCS ANTIMICROBIAL ACTIVITY

The remaining antimicrobial activity against *S typhimurium* SL1344 in both treated samples was determined by the antimicrobial assay described above.

Ammonium sulphate precipitation was conducted by adding solid ammonium sulphate to the bifidobacteria-SCS with stirring until the solution reached 60% saturation. This solution was kept at 4°C overnight to allow complete precipitation of the protein and then centrifuged at 10 000 *g* for 15 minutes. Activity was determined in the pellet resuspended in sterile PBS.

The lipophilic fraction was extracted from bifidobacteria-SCS with chloroform-methanol (1:1, vol/vol). The resulting chloroform layer was dried under nitrogen stream and the lipophilic fraction was resuspended in sterile PBS to test activity.

Estimation of the molecular weight was conducted by dialysis of the bifidobacteria-SCS with Spectra/Por membrane tubing (The Spectrum Companies, Gardena, California, USA), with a molecular weight cut off of 3500.

CELL CULTURE

We used the cultured human colonic adenocarcinoma Caco-2 cell line,[33] which spontaneously differentiates in culture expressing characteristics of the mature enterocyte of the small intestine.[34] Caco-2 cells were routinely grown in Dulbecco modified Eagle's minimal essential medium (25 mM glucose) (Eurobio, Paris, France), supplemented with 20% fetal calf serum (Boehringer, Mannheim, Germany) and 1% non-essential amino acids. Cells were seeded in six well Corning tissue culture plates (Corning Glass Works, Corning, New York, USA) at a concentration of 10^5 cells/cm^2. For maintenance purposes, cells were passaged weekly using 0.25% trypsin in Ca^{2+} Mg^{2+} free PBS containing 0.53 mM EDTA. Maintenance of cells and all experiments were carried out at 37°C in a 10% CO_2/90% air atmosphere. Differentiated cells were used for adherence assays at late post-confluence (15 days in culture).

INFECTION OF CULTURED CELLS BY *S TYPHIMURIUM*

The cell infection assay was conducted as previously reported.[17 30–32 35 36] Briefly, prior to infection, the Caco-2 monolayers were washed twice with PBS. *S typhimurium* SL1344 were suspended in the culture medium and a total of 2 ml (10^7 or 10^8 cfu/ml as mentioned) of this suspension were added to each well of the tissue culture plate. The plates were incubated for one hour at 37°C in 10% CO_2/90% air and then washed three times with sterile PBS. *S typhimurium* internalisation was determined by quantitative determination of bacteria

located within the infected monolayers using the aminoglycoside antibiotic assay. After incubation, monolayers were washed twice with sterile PBS and incubated for one hour in a medium containing gentamicin 50 μg/ml. Bacteria that adhered to the cell brush border were rapidly killed, whereas those located within the cells were not. The infected monolayers were washed with PBS to remove the killed bacteria. The monolayers were lysed with sterilised H_2O. Appropriate dilutions were plated on trypticase soy agar to determine the number of viable intracellular bacteria by bacterial colony counts. Each assay was conducted in triplicate with three successive passages of Caco-2 cells.

Inhibition of *S typhimurium* SL1344 invasion within Caco-2 cell by MRS or bifidobacteria-SCS was examined as previously described.[17 30 31 35 36] Before cell infection, the pathogen (10^8 cfu/ml) was preincubated with MRS or bifidobacteria-SCS (2.5-fold concentrated and adjusted to pH 4.5) for one hour at 37°C. After centrifugation (5500 *g*, 10 minutes at 4°C), bacteria were washed with PBS and resuspended in Caco-2 cell culture medium. Contact between the cells and the MRS or SCS treated *S typhimurium* was for one hour at 37°C. Determination of viable intracellular *S typhimurium* was conducted as described above.

Activity of MRS or bifidobacteria-SCS against intracellular *S typhimurium* was determined using the preinfected Caco-2 as previously described.[31] Cells were infected by *S typhimurium* SL1344 (10^8 cfu/ml) for one hour at 37°C. After washing the infected cells with PBS twice, the extracellular bacteria were killed by gentamicin (50 μg/ml, one hour at 37°C) and cells were washed with PBS to remove the killed bacteria. MRS or bifidobacteria-SCS (2.5-fold concentrated and adjusted at pH 4.5) were added apically to the preinfected cells and incubated for one hour at 37°C. Determination of viable intracellular *S typhimurium* was conducted as described above.

ACTIVITY OF BIFIDOBACTERIUM STRAINS AGAINST *S TYPHIMURIUM* C5 INFECTION IN AXENIC MICE

Antimicrobial activity of bifidobacterium strains was examined in vivo using the protocol previously used to determine anti-salmonella activity of *Lactobacillus* in germ free mice.[32 36] *S typhimurium* C5 strain was grown in Luria broth for 18 hours at 37°C. The culture was harvested in PBS. Viable bacteria were numbered after plating suitable dilutions on TSA and incubation at 37°C for 18 hours. Inoculation of *S typhimurium* C5 in germfree or monoassociated mice was as follows: a single dose of 2×10^6 cfu/mouse was given to the animals, deprived of water since the day before, in bottled water. Monoxenic mice were germfree C3H/He/Oujco mice (six mice per group) inoculated with bifidobacterium strains as a single dose of a 100-fold diluted fresh culture in bottled water, one week before challenge with C5.

DETERMINATION OF BIFIDOBACTERIA COLONISING THE INTESTINAL TRACT OF MONOXENIC MICE

Germ free animals (Iffa Credo, L'Arbresle 69, France) were adult female C3H/He/Oujco mice of 7–8 weeks of age. They were housed and fed in accordance with the relevant national legislation. Germ free mice (Cesal, Orléans, France) were reared in Trexler type isolators fitted with a rapid transfer system (La Calhène, Vélizy Villacoublay, France). They were fed ad libitum a commercial diet RO3 (UAR, Villemoisson/Orge, France) irradiated at 40 kGy and autoclaved demineralised water, or a non-irradiated diet, respectively.

Bifidobacterium strains were inoculated in germ free C3H/He/Oujco mice (six mice per group) as a single dose of a 100-fold diluted fresh culture in bottled water. One week before challenge, monoassociated gnotobiotic mice were killed by cervical elongation. The contents of the stomach, each part of the small intestine (the small intestine was separated into three equal parts: SI-1, SI-2, and SI-3) and the caecum were sampled. To determine the level of bifidobacteria associated with the tissue in different parts of the gastrointestinal tract, the stomach, the three parts of the small intestine, colon, and caecum were sampled and washed eight times with sterilised PBS. The tissues were weighed, mixed with 1 ml of PBS by Ultraturax for two minutes, and then diluted 10-fold. Counts of bifidobacteria were obtained by plating 0.1 ml of each 10-fold serial dilution on MRS agar pH 5.4. The plates were incubated at 37°C for 48 hours under anaerobic conditions (Gaspack H_2+CO_2). Bacterial counts of bifidobacteria are given per gram of content or tissue.

Results

ANTIBACTERIAL ACTIVITY IN VITRO

Fourteen human bifidobacterium strains isolated from infant stools were examined for their antimicrobial activity (table 1). For this purpose, the *S typhimurium* strain SL1344 was

Table 1 Activity of bifidobacterium strains isolated from resident infant human gastrointestinal microflora against the S typhimurium SL1344 strain

Strain	*Viable S typhimurium (log cfu/ml)*
Control *S typhimurium* SL1344	8.0
Bifidobacterium strain	
Viv4	6.5 (0.7)
Viv5	7.5 (0.2)
CA1	3.0 (0.4)**
CA7	6.3 (0.6)
Jo6	6.9 (0.2)
Jo7	7.5 (0.2)
Br13	6.9 (0.2)
Br14	5.8 (0.6)
Br15	6.5 (0.5)
F9	2.1 (0.2)**
Cyn13	7.6 (0.4)
Lara3	7.0 (0.3)
Ana1	7.2 (0.4)
Ana2	5.8 (0.6)

[1]Experimental conditions are described in materials and methods. *S typhimurium* SL1344 (10^8 cfu/ml) was subjected for three hours at 37°C to bifidobacterium-SCS treatment.
Results are presented as mean (SEM) obtained from 3–4 experiments, each in triplicate.
Statistical analysis with Student's *t* test, **p<0.01 compared with control.

Table 2 Effect of infant bifidobacterium strains on the viability of Gram positive and Gram negative pathogens

	Viable bacteria (log cfu/ml)			
	1 hour of contact		*3 hours of contact*	
Strain[1]	*CA1*	*F9*	*CA1*	*F9*
E coli	7.1 (0.2)	6.8 (0.2)	2.1 (0.2)**	2.3 (0.3)**
S flexneri	6.9 (0.2)	7.1 (0.2)	6.7 (0.5)	6.3 (0.5)
K pneumoniae	5.1 (0.2)**	6.5 (0.2)	2.0 (0.2)**	2.0 (0.2)**
P aeruginosa	2.1 (0.2)**	2.1 (0.2)**	2.0 (0.3)**	2.0 (0.3)**
L monocytogenes	6.9 (0.2)	7.0 (0.2)	5.2 (0.4)**	5.0 (0.4)**
Y pseudotuberculosis	7.0 (0.2)	7.0 (0.2)	2.4 (0.2)**	2.7 (0.2)**
S aureus	5.1 (0.2)**	5.1 (0.2)**	2.6 (0.2)**	2.5 (0.2)**
Streptococcus D	7.1 (0.2)	7.1 (0.2)	7.2 (0.2)	7.2 (0.4)
C difficile	7.4 (0.2)	7.4 (0.2)	7.4 (0.2)	7.5 (0.3)

[1]Control for each pathogen: 10^8 cfu/ml. Experimental conditions are described in materials and methods.
Results are presented as mean (SEM) obtained from 3–4 experiments, each in triplicate.
Statistical analysis using a Student's *t* test, **p<0.01 compared with control.

chosen as an indicator. *S typhimurium* SL1344 treated with bifidobacteria-SCS were examined. Only two bifidobacteria-SCS (CA1 and F9) showed high antibacterial activity as a 5–6 log decrease in *S typhimurium* viability was found. MRS (control) showed no activity.

The sensitivity of other pathogens to CA1-SCS and F9-SCS was examined. The viability of all microorganisms was verified after one and three hours of incubation with bifidobacteria-SCS (table 2). The viability of *Streptococcus* spp group D, *S flexneri*, and *C difficile* was not affected at any time points. The viability of *L monocytogenes* was not affected after one hour of contact and was affected to a less degree after three hours of contact (3 log decrease). *E coli*, *K pneumoniae*, *Y pseudotuberculosis*, or *S aureus* viability was not affected or affected to a less degree after one hour of contact, but in contrast was greatly decreased after three hours of contact (5 to 6 log decrease). The viability of *P aeruginosa* was greatly decreased at both times (6 log decrease).

The characteristics of the antibacterial activity of the CA1 and F9 bifidobacteria-SCSs were examined and the *S typhimurium* SL1344 strain was chosen as an indicator (table 3). Precipitation of proteins present in the bifidobacteria-SCSs with ammonium sulphate did not affect activity. Activity was found in the lipophilic fraction extracted from the bifidobacteria-SCSs with chloroform-methanol (1:1). After dialysis of the bifidobacteria-SCSs (molecular weight cut off 3500), the activity disappeared (100% decrease in activity).

Table 3 Characteristics of the bifidobacteria-SCS antibacterial activity

Treatment		*Viable S typhimurium (log CFU/ml)*
Inoculum *S typhimurium*		7.3 (0.2)
Control CA1-SCS		2.0 (0.2)**
Control F9-SCS		2.2 (0.4)**
Ammonium sulphate precipitation[1]	CA1-SCS	7.1 (0.5)
Ammonium sulphate precipitation[1]	F9-SCS	6.8 (0.4)
Chloroform-methanol extraction[2]	CA1-SCS	5.1 (0.2)**
Chloroform-methanol extraction[2]	F9-SCS	2.4 (0.3)**
Dialysis[3]	CA1-SCS	6.9 (0.4)
Dialysis[3]	F9-SCS	6.9 (0.4)

Experimental conditions are described in materials and methods.
Results are presented as mean(SEM) obtained from three experiments.
[1]Activity in pellet obtained by ammonium sulphate precipitation.
[2]Activity in the chloroform fraction after methanol-chloroform extraction.
[3]Activity in the SCS after dialysis with Spectra/Por membrane (molecular weight cut off 3500).
Statistical analysis with Student's *t* test, **p<0.01 compared with inoculum.

Table 4 Activity of the infant bifidobacterium strains against the S typhimurium SL1344 strain infecting the human fully differentiated enterocyte-like Caco-2 cells in culture

Experimental condition	*Viable intracellular S typhimurium (log cfu/ml)*
Pretreated *S typhimurium*[1]	
Control	6.2 (0.2)
MRS treated	5.8 (0.4)
CA1-SCS treated	2.0 (0.2)*
F9-SCS treated	2.4 (0.3)*
Preinfected Caco-2 cells[2]	
Control	7.3 (0.3)
MRS treated	6.5 (0.4)
CA1-SCS treated	4.3 (0.3)*
F9-SCS treated	4.2 (0.2)*

Experimental conditions are described in materials and methods.
Results are presented as mean (SEM) obtained from 18 cell monolayers per group.
*Statistical analysis with Student's *t* test, p>0.01 compared with control.
[1]Prior to cell infection, *S typhimurium* SL1344 (10^8 cfu/ml) was subjected for one hour at 37°C to MRS or bifidobacterium-SCS treatment. After one hour of bifidobacterium-SCS treatment, 10^7 cfu/ml *S typhimurium* remained viable. Consequently, control cells were infected with 10^7 cfu/ml *S typhimurium*.
[2]Cells were infected with 10^8 cfu/ml *S typhimurium*.

ACTIVITY AGAINST *S TYPHIMURIUM* INFECTING THE CULTURED HUMAN INTESTINAL Caco-2 CELLS

Activity of CA1-SCS and F9-SCS against *S typhimurium* strain SL1344 infecting the differentiated enterocyte-like Caco-2 cells[24] was examined using two experimental conditions (table 4).

When *S typhimurium* (10^8 cfu/ml) were subjected to CA1-SCS or F9-SCS for one hour at 37°C prior to the adhesion assay, 10^7 cfu/ml bacteria remained viable. When 10^7 cfu/ml *S typhimurium* were incubated with the Caco-2 cells for one hour at 37°C, 10^6 cfu/ml were found intracellularly. A highly significant decrease in cell entry (4 log decrease) of the CA1-SCS or F9-SCS pretreated *S typhimurium* within Caco-2 cells was observed compared with the untreated *S typhimurium*. As a control, *S typhimurium* (10^8 cfu/ml) was subjected to MRS for one hour at 37°C prior to the adhesion assay. No change in *S typhimurium* viability (not shown) or *S typhimurium* cell entry (table 4) within Caco-2 cells was observed for MRS pretreated *S typhimurium* compared with untreated *S typhimurium*. This result demonstrates that CA1-SCS or F9-SCS inhibited Caco-2 cell infection by *S typhimurium*.

The activity of CA1-SCS or F9-SCS was examined in Caco-2 cells preinfected for one hour at 37°C with *S typhimurium* SL1344 (table 4). A highly significant decrease in viable numbers of intracellular *S typhimurium* was observed when the preinfected Caco-2 cells were exposed to CA1-SCS or F9-SCS (3 log decrease). In contrast, MRS, used as a control, was inactive. This result demonstrates that CA1-SCS or F9-SCS treatment kills intracellular *S typhimurium*.

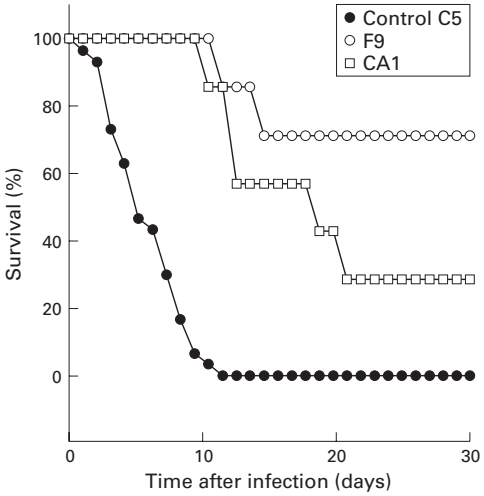

Figure 1 Protective effect of human infant bifidobacterium CA1 and F9 strains established in germ free C3H/He/Oujco mice against S typhimurium lethal infection. Experimental conditions are described in materials and methods.

ANTIBACTERIAL ACTIVITY AGAINST *S TYPHIMURIUM* INFECTING THE GERM FREE MOUSE MODEL

To investigate if CA1 and F9 bacteria protect germ free mice against *Salmonella* infection, CA1 and F9 monoassociated germ free C3H/He/Oujco mice were infected with *S typhimurium* C5. As shown in fig 1, in germ free C5 infected mice, the first mortality was observed four days after infection and 100% mortality was observed 11 days after infection. A highly significant delayed mortality was observed in CA1 and F9 monoassociated mice infected with *S typhimurium* C5 compared with germ free C5 infected mice ($p<0.01$). Indeed, the first mortality occurred at 11–12 days after infection in the CA1 or F9 monoassosiated C5 infected mice. Moreover, 30% and 75% of CA1 and F9 monoassociated C5 infected mice survived at 30 days after infection, respectively.

Table 5 Distribution of infant bifidobacterium colonising the intestine of germ free C3H/He/Oujco mice

Tissue	*CA1*	*F9*
Stomach		
Content	6.1 (0.4)	6.3 (0.9)
Tissue	5.2 (0.4)	4.6 (0.4)
SI-1*		
Content	3.7 (0.9)	5.2 (0.9)
Tissue	3.6 (0.4)	2.8 (0.2)
SI-2*		
Content	4.7 (0.2)	5.0 (0.2)
Tissue	4.4 (0.4)	3.0 (0.3)
SI-3*		
Content	5.8 (0.2)	6.0 (0.2)
Tissue	4.3 (0.2)	3.6 (0.2)
Caecum		
Content	9.0 (0.3)	9.6 (0.2)
Tissue	7.5 (0.2)	6.3 (0.2)
Colon		
Content	8.6 (0.2)	8.7 (0.2)
Tissue	6.8 (0.2)	5.6 (0.2)

Experimental conditions are described in materials and methods.
Levels of bifidobacteria colonising different parts of the intestine in germ free C3H/He/Oujco mice inoculated with a single dose of human infant or adult bifidobacteria. Population of bifidobacteria was determined seven days post-administration in the intestinal content and in washed tissue.
Results are presented as cfu/g of content or cfu/g of tissue (mean (SEM)) obtained from 5–6 mice per group.
*Small intestine (SI) was separated into three equal parts : SI-1, SI-2, and SI-3.

The relative area under the curve showed a large increase in CA1 and F9 monoassosiated C5 infected mice (107 and 147, respectively) compared with germ free C5 infected mice (31).

To examine the levels of bifidobacteria colonising the intestine of mice, germ free C3H/He/Oujco mice received human infant bifidobacteria as a single dose. Seven days after administration, bifidobacteria were established in the intestine (table 5). A population of 4–6 log cfu/g of content was observed in the stomach and three parts of the small intestine for infant CA1 and F9 strains. A similarly high number of populations (9 log cfu/g of content) was found in the caecum and colon for the two infant strains. The level of bifidobacteria associated with the tissue was determined after the tissues were washed eight times with sterilised PBS. A population of 3–5 log cfu/g of tissue was observed in tissues of the stomach and three parts of the small intestine for infant CA1 and F9 strains. An identical population of 6–7 log cfu/g of tissue was found in the caecum and colon for the two infant strains.

Discussion

Important to human health, the gastrointestinal microflora contains a substantial and complex collection of microorganisms forming a biologically pivotal component of the host body.[11 12] This microflora is composed of different species of microorganisms. Some interactions between species have been observed. The microflora exerts properties which are potentially damaging or health promoting for the host.[11] A long established concept is that of beneficial and harmful species. Among components of the microflora, it has been suggested that bifidobacteria play a role in acting as a barrier against colonisation of the gastrointestinal tract by pathogenic bacteria. In addition to bifidobacteria, lactobacilli in particular have been examined concerning their role in the "barrier effect" against pathogens. Recent reports have documented that lactobacilli, a minor genus of the gut microflora, inhibit attachment of pathogens onto cultured uroepithelial[37 38] and intestinal[39 40] cells, and mucus.[41] Moreover, reports have appeared showing that lactobacilli in gnotobiotic mice and continuous flow cultures can compete with *Escherichia coli* in the stomach and small intestine, whereas *Clostridia* have been found to control *E coli* in the large intestine.[42] Lactobacilli monoassociated mice[30 36] and conventional mice treated with lactobacilli[43] are protected against infection. Others reports show a potential for bifidobacteria, isolated from human adult stools, in inhibiting binding of pathogens in an in vitro model.[20]

Our results presented here indicate that: (i) not all bifidobacterium strains resident in the infant microflora have antibacterial activity; (ii) the two infant bifidobacterium strains CA1 and F9 that developed antibacterial activity in vitro can colonise the digestive tract of germ free mice; (iii) the established infant CA1 and F9 bacteria exert efficient antimicrobial activity against *S typhimurium* infection in mice. The

mechanism by which bifidobacteria develop antimicrobial properties remains to be elucidated. Several reports have indicated that bifidobacteria could stimulate the immune system.[15 18 21] For example, bifidobacterium strains increase the resistance of rats to salmonella infection.[44] *B breve* YIT4064 enhances antigen specific IgA antibody directed against rotavirus in the mouse mammary gland protecting pups that receive milk against rotavirus challenge.[18] Another report showed that a colonisation resistance mechanism against *S typhimurium* in human faecal bacteria associated mice inoculated with a mixture of bacteria isolated from the faeces of human breast fed infants containing *B bifidum* was related to lowering of the pH level and the presence of short chain fatty acids in the caecal contents.[19] Another mechanism of action has been proposed as a *B infantis* strain developed broad spectrum antimicrobial properties through production of antimicrobial compounds, unrelated to acid production, which inhibited the growth of pathogens.[16] We have recently provided evidence that selected bifidobacterium strains isolated from adult human stools inhibit binding of human enteropathogens onto cultured enterocyte-like Caco-2 cells.[17] Our results presented here and related to the antimicrobial activity of infant bifidobacterium strains are consistent with one of the mechanisms of action dependent on the production of antimicrobial compounds, as previously hypothesised.[16] Indeed, we have provided evidence that the activity of the strains CA1 and F9 in vitro results from antimicrobial compounds present in the spent culture supernatants, suggesting that they are secreted. Interestingly, Fujiwara and colleagues[20] recently described a proteinaceous factor(s) produced by *Bifidobacterium longum* SBT 2928, with a molecular weight of at least 100 000, which inhibited adherence of enterotoxigenic *E coli* strain Pb176 expressing the colonisation factor adhesion II to the gangliotetrasylceramide GA1 molecule in vitro.[20] We found that CA1 and F9 bifidobacteria produced an antibacterial lipophilic factor(s) with a molecular weight estimated as lower than 3500. Ibrahim and Bezkorovainy[45] reported that organic acids of bifidobacteria serve as anti-infectious agents. The characteristics of bifidobacteria antimicrobial factor(s) resemble those of the antibacterial factor(s) produced by several lactobacilli strains[46] and in particular those isolated from human stools and secreting antimicrobial compounds such as *Lactobacillus casei rhamnosus* GG,[36 47] *L johnsonii* LA1,[30] and *L acidophilus* LB,[31 39 40] which inhibit *S typhimurium* infection both in vitro and in vivo.

Over the past 10 years, evidence has accumulated that there is a non-immune system of defence in the intestine.[48 49] By continual release of antibiotic proteins such as defensins and cryptdins, and enzymes such as lysozyme and phospholipase A_2 with antimicrobial activity, specialised cells of the intestinal epithelium may influence the extracellular environment and contribute to mucosal barrier function. In parallel with the host cell non-immune system of defence, bacteria of the resident gut microflora also produce antimicrobial substances.[50] The results presented here demonstrate that other species of the endogenous microflora such as bifidobacteria could participate in the host defence against potential pathogenic microorganisms by producing antimicrobial substances.

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